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(54) Title: NOVEL VIRAL VECTORS HAVING ENHANCED EFFECTIVENESS WITH DRAMATICALLY REDUCED VIRU-
LENCE

(57) Abstract: The present invention provides recombinant vaccinia virus from which the region encoding the N-terminal 83 amino
acids of the E3L gene product has been deleted. Compositions comprising the recombinant vaccinia virus are also provided.

**NOVEL VIRAL VECTORS HAVING ENHANCED
EFFECTIVENESS WITH DRAMATICALLY REDUCED VIRULENCE**

SPECIFICATION

STATEMENT REGARDING FEDERALLY FUNDED RESEARCH

5 Financial assistance for this project was provided by the U.S. Government through the National Institutes of Health under grant number CA-4865409 and the United States Government may own certain rights in this invention.

BACKGROUND OF THE INVENTION

 Vaccinia virus is a member of the poxvirus family of DNA viruses.
10 Poxviruses including vaccinia virus are extensively used as expression vectors since the recombinant viruses are relatively easy to isolate, have a wide host range, and can accommodate large amounts of DNA.

 The vaccinia virus genome contains nonessential regions into which exogenous DNA can be incorporated. Exogenous DNA can be inserted into the
15 vaccinia virus genome by well-known methods of homologous recombination. The resulting recombinant vaccinia viruses are useful as vaccines and anticancer agents.

 The use of vaccinia virus recombinants as expression vectors and particularly as vaccines and anticancer agents raises safety considerations associated with introducing live recombinant viruses into the environment. Virulence of vaccinia
20 virus recombinants in a variety of host systems has been attenuated by the deletion or inactivation of certain vaccinia virus genes that are nonessential for virus growth. However, there remains a need in the art for the development of vectors that have reduced pathogenicity while maintaining desirable properties of wild-type virus, such as host range, and active protein synthesis of a desired gene product.

SUMMARY OF THE INVENTION

The present invention provides a recombinant vaccinia virus from which the region encoding an N-terminal portion of the E3L gene product has been deleted. In a preferred embodiment, the region encoding the N-terminal 83 amino acids of the E3L gene product has been deleted. The recombinant vaccinia virus of
5 the invention may further comprise exogenous DNA.

The present invention further provides a composition comprising the recombinant vaccinia virus of the invention and a carrier.

DETAILED DESCRIPTION OF THE INVENTION

10 The vaccinia virus E3L gene codes for double-stranded RNA binding proteins, and has been shown to be necessary for the vaccinia virus interferon-resistant phenotype. The E3L gene product of the vaccinia virus is a 190 amino acid polypeptide. Amino acids 118 to 190 have been implicated in dsRNA binding, as disclosed by Kibler et al. (1997) *J. Virol.* 71: 1992, incorporated herein by reference.

15 The present invention provides a recombinant vaccinia virus from which the region of the viral genome encoding an N-terminal portion of the E3L gene product has been deleted. An N-terminal portion of the E3L gene product is defined herein as including at least amino acids 1 through 37 of the E3L gene product. Amino acid numbering as used herein is adopted from Goebel et al. (1990) *Virology* 179:
20 247-66, 577-63, the disclosure of which is incorporated herein by reference. An N-terminal portion of the E3L gene product as defined herein may encompass the region from the N-terminus (amino acid 1) up to and including amino acid 117. Accordingly, a region encoding at least 37, and as many as 117, contiguous N-terminal amino acids of the E3L gene product is deleted from the recombinant vaccinia virus of the present
25 invention.

In a preferred embodiment, the region of the viral genome encoding the N-terminal 83 amino acids of the E3L gene product has been deleted. In this preferred

embodiment, the recombinant vaccinia virus of the present invention contains a nucleic acid fragment encoding amino acids 84-190 of the E3L gene product instead of the gene encoding amino acids 1-190 of the E3L gene product at the E3L locus of vaccinia virus.

5 The recombinant vaccinia virus may further contain exogenous, i.e., nonvaccinia virus, DNA. Exogenous DNA may encode any desired product, including for example, an antigen, an anticancer agent, or a marker or reporter gene product. The recombinant vaccinia virus may further have deletions or inactivations of nonessential virus-encoded gene functions. Nonessential gene functions are those
10 which are not required for viral replication in a host cell.

 The recombinant vaccinia virus of the present invention may be constructed by methods known in the art, and preferably by homologous recombination. Standard homologous recombination techniques utilize transfection with DNA fragments or plasmids containing sequences homologous to viral DNA,
15 and infection with wild-type or recombinant vaccinia virus, to achieve recombination in infected cells. Conventional marker rescue techniques may be used to identify recombinant vaccinia virus. Representative methods for production of recombinant vaccinia virus by homologous recombination are disclosed by Piccini et al. (1987) Methods in Enzymology 153:545, the disclosure of which is incorporated herein by
20 reference.

 For example, the recombinant vaccinia virus of a preferred embodiment of the present invention may be constructed by infecting host cells with vaccinia virus from which the E3L gene has been deleted, and transfecting the host cells with a plasmid containing a nucleic acid encoding amino acids 84-190 of the
25 E3L gene product flanked by sequences homologous to the left and right arms that flank the vaccinia virus E3L gene. The vaccinia virus used for preparing the recombinant vaccinia virus of the invention may be a naturally occurring or engineered strain. Strains useful as human and veterinary vaccines are particularly preferred and are well-known and commercially available. Such strains include
30 Wyeth, Lister, WR, and engineered deletion mutants of Copenhagen such as those disclosed in U.S. Patent 5,762,938, which is incorporated herein by reference.

Recombination plasmids may be made by standard methods known in the art. The nucleic acid sequences of the vaccinia virus E3L gene and the left and right flanking arms are well-known in the art, and may be found for example, in Earl et al. (1993) in Genetic Maps: locus maps of complex genomes, O'Brien, ed., Cold Spring Harbor Laboratory Press, 1.157 the disclosure of which is incorporated by reference, and Goebel et al. (1990), supra. The amino acid numbering used herein is adopted from Goebel et al. (1990), supra. The vaccinia virus used for recombination may contain other deletions, inactivations, or exogenous DNA as described hereinabove.

Following infection and transfection, recombinants can be identified by selection for the presence or absence of markers on the vaccinia virus and plasmid. Recombinant vaccinia virus may be extracted from the host cells by standard methods, for example by rounds of freezing and thawing.

The resulting recombinant vaccinia virus may be further modified by homologous recombination to provide other deletions, inactivations, or to insert exogenous DNA.

It has been discovered in accordance with the present invention that a recombinant vaccinia virus having a deletion of the DNA encoding a N-terminal portion of the E3L gene product, and preferably amino acids 1-83 of the E3L gene product, maintains viral replication, protein synthesis, inteferon-resistance and cell tropism that is indistinguishable from wild-type virus, but has remarkably reduced pathogenicity in mice relative to wild-type vaccinia virus of the same strain.

The present invention further provides a composition comprising the recombinant vaccinia virus of the invention and a carrier. The term carrier as used herein includes any and all solvents, diluents, dispersion media, antibacterial and antifungal agents, microcapsules, liposomes, cationic lipid carriers, isotonic and absorption delaying agents, and the like.

The recombinant vaccinia viruses and compositions of the present invention may be used as expression vectors in vitro for the production of recombinant gene products, or as delivery systems for gene products, as human or veterinary vaccines, or anticancer agents. Such utilities for recombinant vaccinia viruses are known in the art, and disclosed for example by Moss (1996) "Poxviridae:

The Viruses and Their Replication" in Virology, Fields et al., eds., Lippincott-Raven, Philadelphia, pp. 2637-2671, incorporated herein by reference.

All references cited herein are incorporated in their entirety.

The following nonlimiting examples serve to further illustrate the
5 invention.

Example 1

Construction of Recombinant Vaccinia Virus

The plasmid pMPE3ΔGPT (described by Kibler et al. (1997) J. Virol.
71:1992, incorporated herein by reference) was used for recombining a truncated
10 E3L gene into the E3L locus of the WR strain of vaccinia virus. The recombination
plasmid pMPE3ΔGPT is a derivative of pBSIISK (Stratagene, La Jolla, CA) that has
had the β-galactosidase sequences deleted, and that contains sequences homologous to
the left and right arms flanking the vaccinia virus E3L gene, but that lacks the E3L
gene itself. The recombination plasmid contains the E. coli gpt gene outside the E3L
15 flanking arms and thus allows for selection of transfected cells by treatment with
mycophenolic acid (MPA).

The Aat II (blunt-ended) Sal I fragment of E3L was subcloned into the
pGEM3-5T vector (described by Chang et al. (1993) Virology 194: 537, the disclosure
of which is incorporated by reference) and subsequently cloned into the pMPE3ΔGPT
20 recombination plasmid using Bam HI and Hind III restriction sites. The E3L fragment
encodes amino acids 84-190 of the E3L gene product as numbered by Goebel et al
(1990), supra, and has a deletion of the DNA encoding the N-terminal amino acids 1-
83. The plasmid resulting from the cloning of the E3L fragment into pMPE3ΔGPT is
designated pMP-Δ83N.

25 In vivo recombination was performed in baby hamster kidney (BHK)
cells. Subconfluent BHK cells were simultaneously infected with the WR strain of
vaccinia virus deleted of the E3L gene (WRΔE3L) at a multiplicity of infection (MOI)
of 5 and transfected with 1 μg of pMP-Δ83N using Lipofectace (Gibco BRL).
WRΔE3L was prepared by replacing the E3L gene from the WR strain of vaccinia

virus with the lacZ gene, by homologous recombination with pMPE3ΔGPT in which the lacZ gene was inserted between the E3L flanking arms.

Thirty hours post infection, the cells were harvested and recombinant virus was subjected to selection as follows. Virus was extracted from infected/transfected cells by three rounds of freezing and thawing and used to infect confluent BHK cells that had been pretreated for six hours with MPA selection medium (Modified Eagle Medium (MEM) containing 10% fetal bovine serum (FBS), 10μg/ml mycophenolic acid, 250μg/ml xanthine, 15μg/ml hypoxanthine). Following infection, cells were overlaid with MPA selection medium. At 24-72 hours post infection, plaques were visible and dishes were overlaid with MPA selection medium containing 0.5% molten agarose and 0.4μg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside). Blue plaques were isolated four to six hours after X-gal overlay. Two more rounds of MPA selection were performed on the isolated blue plaques.

Resolution of the *in vivo* recombination occurs when the MPA selection medium is removed, resulting in either recovery of the original virus, WRΔE3L (containing lacZ in the E3L locus) or a recombinant virus containing the Δ83N deletion of E3L in the E3L locus. MPA-resistant blue plaques were used to infect untreated rabbit kidney RK13 cells. At 24-48 hours post infection, dishes were overlaid with MEM medium containing 0.5% molten agarose and 0.4μg/ml X-gal. Both blue and clear plaques were visible. Blue plaques indicate resolution of WRΔE3L with lacZ in the E3L locus. Clear plaques indicate resolution of virus containing the Δ83N deletion of E3L in the E3L locus.

Two more rounds of infections with clear plaques were performed to purify plaques containing WRΔ83N. Recombinant virus was amplified in RK13 cells.

Nucleic acid sequencing was used to confirm that the Δ83N fragment of E3L of plasmid pMP-Δ83N was present in the recombinant virus. Viral DNA was extracted from cells infected by WRΔ83N. Infected cells were freeze-thawed three times, followed by a thirty second sonication. Cell debris was removed by centrifugation at 700 x g for ten minutes. Nucleic acid was obtained by

phenol/chloroform extraction of the supernatant, and PCR was performed using primers to the E3L flanking arms. The PCR reaction products were subjected to agarose gel electrophoresis. DNA was extracted from the band of interest and DNA sequencing was performed. The $\Delta 83N$ fragment was identified by sequence
5 comparison to the plasmid DNA sequence of pMP- $\Delta 83N$.

Example 2

Host Range and Interferon Resistance of WR, WR $\Delta 83N$ and WR $\Delta 3L$

Wild-type vaccinia virus of the WR strain (WR) and variants WR $\Delta E3L$
10 and WR $\Delta 83N$ as described in Example 1 were assessed for interferon resistance as follows.

RK13 cells were set down in six well tissue culture dishes at 70-80% confluency. Cells were treated with varying concentrations of rabbit interferon alpha (0-1000 U/ml) for sixteen hours prior to infection. Cells were infected with
15 approximately 100 plaque forming units (pfu) of WR, WR $\Delta E3L$ or WR $\Delta 83N$ virus. Dishes were stained with crystal violet 24 hours post infection and plaques were counted.

WR $\Delta E3L$ exhibited interferon sensitivity (as measured by plaque reduction) at a concentration of 10 Units/ml of interferon, whereas WR $\Delta 83N$ and WR
20 did not exhibit interferon sensitivity at 10 or 100 Units/ml, but both showed plaque reduction at a concentration of 1000 Units/ml.

The foregoing results indicate that WR $\Delta E3L$ is sensitive to the effects of interferon, and that WR $\Delta 83N$, like WR, exhibits an interferon-resistant phenotype.

WR, WR $\Delta E3L$ and WR $\Delta 83N$ were assayed for host range as follows.
25 Six-well tissue culture dishes containing RK13 cells or HeLa cells were set down simultaneously at 70-80% confluency. Both cell types were infected with equal dilutions of virus, and 24-48 hours post infection cells were stained with crystal violet and plaques were counted for each cell type. A comparison was made by determining the efficiency of plaquing (number of plaques in HeLa cells divided by number of

plaques in RK13 cells) for each virus. The efficiencies of plaquing were: WR: 0.98; WRΔ83N: 1.06; WRΔE3L: <0.01.

These results indicated that WRΔE3L has a restricted host range in that it cannot replicate in HeLa cells but exhibits nearly wild-type replication in RK13 cells. WRΔ83N, like wild-type WR, replicates in RK13 cells and HeLa cells.

The foregoing results show that WR and WRΔ83N are identical with respect to host range and interferon resistance in the cultured cells evaluated, whereas WRΔE3L is sensitive to interferon and has a restricted host range.

Example 3

Virulence of WR, WRΔE3L and WRΔ83N

Virus (WR, WRΔE3L or WRΔ83N) was amplified by infection of RK13 cells until 100% CPE (cytopathic effect) was observed. Cells were scraped and resuspended in 1 mM Tris, pH 8.8. Amplified virus was freeze-thawed three times to release virus from cells. Debris was removed by centrifugation at 700 x g for 10 min. Supernatant was used for mouse infections. Various dilutions of virus in 1 mM Tris, pH 8.8 were used in the experiment to determine LD50.

Three to four week old c57b16 mice were anesthetized by intrafemoral injection of a cocktail of ketamine, acepromazine, and xylazine. Mice were subsequently infected with 10 μ l of virus or a dilution of virus intranasally using a pipetman and gel loading tip. Mice were then replaced in their cages and observed daily for pathogenesis and death.

Intranasal inoculation with WR resulted in death at 10⁴ pfu, whereas no pathogenesis could be detected with WRΔE3L at the highest dose. For inoculation with WRΔ83N, 10⁷ pfu was required for death, indicating that the amino-terminus of E3L is an important determinant for virus virulence.

CLAIMS

1. Vaccinia virus from which the region encoding at least amino acids 1 through 37 of the E3L gene product has been deleted.
- 5 2. Vaccinia virus according to Claim 1 from which the region encoding amino acids 1 through 83 of the E3L gene product has been deleted.
3. Vaccinia virus according to Claim 1 from which the region encoding amino acids 1 through 117 of the E3L gene product has been deleted.
- 10 4. Vaccinia virus according to Claim 1 from which one or more nonessential virus-encoded gene functions have been deleted or inactivated.
5. Vaccinia virus according to claim 1 comprising nonvaccinia virus DNA.
6. Vaccinia virus of Claim 1 which is the WR strain of vaccinia virus.
7. Recombinant vaccinia virus WR Δ 83N.
- 15 8. A composition comprising the vaccinia virus of Claim 1 and a carrier.
9. A composition comprising recombinant vaccinia virus WR Δ 83N and a carrier.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/10948

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12P 21/06; C12N 15/39, 15/64; C07H 21/02, 21/04

US CL : 435/69.1, 91.42, 320.1; 536/23.1, 23.72

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 91.42, 320.1; 536/23.1, 23.72

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WEST 2.0

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E,A	US 6,004,777 A (TARTAGLIA et al.) 21 December 1999, see entire document.	1-9

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

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